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Sources and seasonal dynamics of inoculum for brown spot disease of pear

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Abstract The dynamics of the production of *Stemphylium vesicarium* conidia and *Pleospora allii* ascospores from different inoculum sources on the ground were compared in a model system of a wildflower meadow mainly composed of yellow foxtail, creeping cinquefoil and white clover. The meadow was either inoculated (each October) or not inoculated with a virulent strain of *S. vesicarium*, and either covered or not covered with a litter of inoculated pear leaves. Spore traps positioned a few centimetres above the ground were exposed for 170 7-day periods between October 2003 and December 2006. Ascospores and conidia were trapped in 46 and 25% of samples, respectively. Ascospore numbers trapped from the pear leaf litter were about five times higher than those from the meadow, while conidial numbers were similar from the different inoculum sources. The ascosporic season was very long, with two main trapping periods: December–April, and August–October; the former was most important for the leaf litter, the latter for the meadow. The conidial season lasted from April to November, with 92% of conidia caught between July and September. The fungus persistently colonized the meadow: the meadow inoculated in early October 2003 produced spores until autumn 2006. The present work demonstrates that orchard ground is an important source of inoculum for brown spot of pear. Thus, it is important to reduce inoculum by managing the orchard ground all year long.

Keywords Ascospores · Conidia · Leaf litter · Meadow plants · *Stemphylium vesicarium* · *Pleospora allii*

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Introduction

Brown spot of pear (*Pyrus communis*), caused by *Stemphylium vesicarium* (Ellis and Ellis 1985), is a disease of economic importance in fruit-growing areas of Europe (Llorente and Montesinos 2006). Disease symptoms consist of necrotic lesions on leaves, twigs and fruits. Fruits show small necrotic spots that progressively enlarge and deepen in round-shaped brown areas that can rot; infected fruits are unmarketable. Epidemics usually begin in late spring when fruits are highly susceptible (Montesinos et al. 1995), and increase progressively to reach the maximum just before harvest (Montesinos and

Vilardell 1992) when high spore densities and conducive environmental conditions for infection (Montesinos et al. 1995) can result in 80–90% of infected fruits (Ponti and Laffi 1993).

The disease cycle is characterized by a sexual phase during winter and spring, when the pathogen produces pseudothecia and ascospores of *Pleospora allii*, and an asexual phase in spring to autumn, when the fungus produces the typical conidia of *S. vesicarium*. The role of these two phases in the epidemiology of this disease is not completely understood (Cavanni and Ponti 1994), particularly the sources of inoculum for pear infection (Llorente and Montesinos 2006). Pseudothecia were observed only recently on infected pear leaves on orchard ground (Llorente et al. 2003; Maccaferri et al. 2003). Ascospores are released from February to May or early June (Llorente et al. 2003), but disease symptoms usually first appear from mid-June (Ponti et al. 1982), when few or no ascospores are present in the orchard air (Picco et al. 1996). In this period, conidia are usually abundant and continue to be airborne during the pear-growing season (Rossi et al. 2005b), though sporulation rarely occurs on infected pear tissues (Maccaferri et al. 2003). Recent studies (Rossi et al. 2005a) demonstrated that *S. vesicarium* strains are able to colonize dead tissues of herb plants as saprophytes and to produce abundant ascospores and conidia that are capable of infecting pear. Based on these findings, the life cycle of the fungus was redrawn (Rossi et al. 2005a). Pseudothecia are produced in autumn on both the fallen pear leaves colonised by the pathogen and the saprophytically colonized plant materials of the meadow. Ascospores mature and disperse from winter to spring and may infect pear leaves or, more likely, initiate the saprophytic colonization of the dead material of the meadow. These produce conidia and may act as a source of inoculum for the infections on pear leaves and fruits during the whole season. The pear leaf litter, on the contrary, diminishes progressively and is nearly destroyed in August.

Even though this new hypothesis was readily accepted (Llorente and Montesinos 2006), there is no definitive proof that the orchard ground is the source of airborne inoculum for infections during summer on pear. This study was therefore undertaken with the aim of: (1) investigating the dynamics of airborne spores from the meadow; (2) comparing the production of spores from the pear leaf litter and from the meadow; (3) demonstrating the capability of *S. vesicarium* to saprophytically colonize the dead herbs of the meadow persistently. The work was necessarily carried out in a model system, because the orchard floor is already naturally colonised by *S. vesicarium* and both saprophytic and pathogenic strains coexist.

Materials and methods

Experimental design, plants and fungal materials

Experiments were carried out at the campus of the University of Piacenza (north Italy) on a 60 m^2 15 year-old grassy area naturally converted into a wildflower meadow. At the beginning of the experiment, the meadow was composed of several herb species. Yellow foxtail (*Setaria glauca*), creeping cinquefoil (*Potentilla repens*) and white clover (*Trifolium repens*) were the prevalent plants, all with about 15% frequency, ground ivy (*Gleochoma hederacea*) and dovefoot geranium (*Geranium molle*) were both 8–10% in frequency; Bermuda grass (*Cynodon dactylon*), heal-all (*Prunella vulgaris*), prostrate knotweed (*Polygonum aviculare*), violets (*Viola* spp.), woodsorrel (*Oxalis* sp.), field bindweed (*Convolvolus arvensis*), hawkbeard (*Crepis* sp.), and hawkbit (*Leontodon* spp.) were present at lower frequencies. This study area was chosen because: (1) it was representative of the lawns that usually cover the ground of the pear orchards in the Po Valley, which progressively become wildflower meadows with age; (2) it was 30 km from any source of *S. vesicarium* inoculum from pear orchards. Conditions of temperature (T, in °C), relative humidity (RH, in %) and rainfall (R, in mm) were continuously monitored with electronic equipment (Weather Monitor IITM, Davis Instruments, San Francisco, U.S.A.) sited 50 m from the study area.

Fifteen plots (80×40 cm) were prepared in September 2003, arranged in a square, separated by 1 m wide bands to avoid interplot interference, and maintained until December 2006. No irrigation, manuring or pesticides were applied to the meadow for the entire period of the experiment; the sward was cut when it was about 20 cm in height. A cover made with a metallic netting (2 cm mesh) was placed over each plot to prevent damage due to rodents or birds.

Five treatments were arranged in these plots according to a complete randomized design, with three replicates. Treatments are shown in Table 1. There were combinations of two sources of *S. vesicarium* inoculum: the leaf litter composed by the fallen pear leaves that had been infected during the season and the dead leaves of the groundcover plants saprophytically colonized by the fungus. The leaf litter was formed with artificially inoculated pear leaves. Leaves of cv. Abate Fétel were collected each year just before leaf fall from an orchard at Villanova d'Arda (40 km from the university campus) that had never shown brown spot symptoms. Inoculations were performed on 2 October 2003, 29 September 2004, and 5 October 2005, following the method of Rossi et al. (2005a). Leaves were washed under running tap water for 15 min, autoclaved at 120°C for 20 min, laid on blotting paper, and then inoculated by uniformly distributing with a hand-nebulizer 100 ml of inoculum suspension per 100 g of leaves. After 2 days of incubation at 25°C, 12 h day length and 100% RH, leaves were uniformly placed onto the ground of the plots, 300 g of leaves per plot. The meadow was also inoculated with a hand-nebulizer, by distributing 100 ml of inoculum suspension per plot. Inoculations were performed on 4 October 2003, 1 October 2004, and 7 October 2005, i.e. the same day when the inoculated pear leaves were arranged onto the plot ground.

The inoculum suspension was prepared using the strain ISA94 of *S. vesicarium*, which causes severe disease symptoms on pear leaves (Rossi et al. 2005a). Conidia were collected from 14 day-old fungal colonies grown on V8 (Campbell Ltd., Italy) agar in Petri dishes incubated under fluorescent light at 20°C with 12 h day length. The colonies were dispersed in 10 ml of distilled water using a spatula and the resulting suspension was filtered through a double layer of cheesecloth and adjusted to 1×10^5 conidia ml^{-1}. The germination rate of these conidia was higher than 90% after 2 h of incubation at 25°C.

Table 1 Characteristics of the different inoculum sources for *Pleospora allii* ascospores and *Stemphylium vesicarium* conidia

Type of inoculum source	Inoculated pear leaf litter[a]	Inoculated meadow[b]	Inoculation performed[c]
I	Yes	No	Each year
II	Yes	Yes	Each year
III	No	Yes	In 2003 only
IV	No	Yes	Each year
V	No	No	Never

[a] Pear leaves of cv. Abate Fétel were artificially inoculated and placed onto the meadow 2 days later

[b] The meadow was a grassy area naturally converted into a wildflower meadow.

[c] Inoculations were performed in early October with conidial suspensions of the virulent strain ISA94 of *S. vesicarium*.

Observation of pseudothecia

To study the maturation course of pseudothecia, 20–30 fruiting bodies were excised from leaves of the leaf litter and of the meadow plants, at an interval of 15 days from November to mid-July each year; they were placed onto microscope slides, crushed, stained with 0.1% acid fuchsin in lactophenol, and examined under a microscope. The frequency of pseudothecia with mature ascospores was determined following the method of Prados-Ligero et al. (1998).

Trapping of *S. vesicarium* spores

To determine seasonal dynamics of *S. vesicarium* conidia and *P. allii* ascospores, spore traps were installed in each plot between October 2003 and December 2006. Traps consisted of glass slides (76 mm×26 mm) with the underside coated with a silicon grease (Lanzoni Srl, Bologna, Italy) that were positioned at 3–5 cm above the ground. Glass slides were removed and replaced by new ones every 7 days, for a total of 170 replacements. Four slides were used per plot. Slides were brought to the laboratory and fixed using a jelly solution (Lanzoni Srl, Bologna, Italy). Ascospores and conidia were counted under a microscope in two longitudinal traverses at 100–200 magnification, and numbers of spores cm^{-2} were estimated. The two types of spores were distinguished based on the characters reported by Simmons (1969): size, shape, colour and number of transverse septa. In case of doubt, spores were examined for presence/absence of the basal scar-like zone. All counts were made by the same observer.

Between July and November 2006, additional spore traps were used to collect viable propagules for use in the pathogenicity tests. Glass slides were exposed as previously described for 24 h at weekly

intervals; the trapping surface was covered with a layer (about 1 mm in thickness) of a substrate made with carboxy methyl cellulose (19%), glycerol (27%) and distilled water (46%). After exposure, the slides were brought to the laboratory and the substrate was dispersed in 10 ml of peptonate water. One ml of the resulting suspension was diluted in distilled water (10^{-2}, 10^{-3}, 10^{-4}) and three replicate aliquots (0.1 ml) of each diluted suspension were plated on Potato Dextrose Agar (PDA) medium. After 48 h of incubation at 20°C in the dark, the colonies resembling *Stemphylium* spp. were transferred onto new Petri dishes with PDA, incubated at 20°C, and then identified according to Ellis (1971). Single-spore strains of *S. vesicarium* were obtained from these colonies and maintained.

Pathogenicity of the trapped spores

The bioassay described by Rossi et al. (2005a) was used to verify that the *S. vesicarium* propagules from the different inoculum sources belonged to the strain that had been used for inoculation. The single-spore strains obtained as described previously were tested for pathogenicity in comparison with the ISA94 strain and an uninoculated control. Young leaves of cv. Abate Fétel, a highly sensitive pear variety, were detached from plants grown in a glasshouse and inoculated on the lower leaf surface with 10 µl drops of a conidial suspension prepared as previously described (four replicates, four leaves per replicate, six inoculation drops per leaf). Control leaves were inoculated with water. Leaves were incubated at 25°C, 100% RH and 12 h day length for 14 days, and inspected daily to determine the number of inoculated drops causing necrosis of the leaf tissue. Disease incidence was calculated as a percentage of the inoculation points showing necrosis.

Data analyses

Three years of spore trap data were defined, each consisting of 53 7-day samplings following the inoculations made in October 2003, 2004 and 2005; an additional 11 samples were collected in October to December 2006.

An analysis of variance (ANOVA) of repeated measurements was performed to test differences in the numbers of ascospores and conidia trapped during the 3 years (2003/04 to 2005/06), the four inoculum sources (I–IV of Table 1) and the 53 7-day samplings per year, with 12 replicates (12 spore traps per treatment: three replicate plots, four traps per plot). Before performing the ANOVA, the data were transformed using natural logarithms. Mauchly's Test was applied to test the sphericity of the common covariance matrix. When this test was significant (no sphericity) the degrees of freedom (df) were corrected by the Huynh–Feldt Epsilon to have valid *F*-tests and associated probability values (Everitt and Howell 2005). Means were compared using Fisher's protected least significant difference (LSD) at $P=0.05$.

To study seasonality in spore trappings, each sample (of the 680 samples collected, 170 per inoculum source) was defined as positive (=1), when at least one spore was trapped, or negative (=0), when no spores were trapped. An analysis of similarity for these binary data was applied to test the level of matching in spore trap data between different inoculum sources. Similarity was calculated for each comparison (for instance I versus II) as the ratio between matching pairs of data in a sample and total number of samples, with positive (1 versus 1) and negative (0 versus 0) matches being weighted equally. The Yates chi-square test (χ^2) was used to test for independence in the corresponding 2×2 contingency tables (Everitt and Howell 2005).

To test differences between disease incidences caused by the fungal strains from the different inoculum sources and the strain ISA94, an ANOVA was applied to the apparent rates of disease progression. Apparent rates were calculated as the slope of the regression lines that fits the gompit transformation of disease proportion with time, where gompit is the linearizing transformation of the Gompertz function: $\text{gompit}=-\ln[-\ln(y)]$ (Campbell and Madden 1990). The statistical analyses were performed using SPSS (ver. 11.5, SPSS Inc., Chicago, USA).

Results

Inoculum production by the different sources

Ascospores and conidia were never trapped from the uninoculated meadow (inoculum type V, Table 1); therefore, these data were not included in further analyses.

Numbers of the ascospores trapped were significantly ($P<0.0001$) influenced by the year and the inoculum source, and by their interaction; these accounted for 26.3% of the total variance (Table 2). In the first year (Fig. 1a), the treatments with pear leaves as the inoculum source (I and II) produced five times more ascospores than those with the meadow only (III and IV). The presence of an inoculated meadow in addition to the pear leaf litter (II) did not significantly increase the total number of ascospores compared to the leaf litter alone (I). In the second year (Fig. 1b), the total number of ascospores was less than in the previous year and was not significantly influenced by the inoculum sources. In particular, the meadow inoculated with *S. vesicarium* in autumn 2003 (III) produced a similar number of ascospores to that inoculated in both 2003 and 2004 (IV). Results obtained in the third year (Fig. 1c) were consistent with those found in the first year, but the ascospore numbers were low. Also in this year, total number of ascospores trapped from the meadow inoculated only in autumn 2003 was similar to that from the meadow inoculated for 3 years.

Ascospore numbers were also significantly ($P<0.0001$) influenced by the time of sampling: sampling time and all interactions were significant ($P<0.0001$), accounting for 73.7% of the total variance (Table 2). Between-sampling differences ranged from no ascospores (in 54% of the total samples) to 417 ascospores cm^{-2} in the traps over inoculum source II in the week between 20 and 27 February 2004 (Fig. 3a).

Numbers of conidia were significantly ($P<0.0001$) influenced by the year and the time of sampling, and by their interaction, and this accounted for 6.4, 32.7 and 49.7% of the total variance, respectively. The inoculum source and its interaction with the year were not significant, while interactions between inoculum sources and sampling time were significant (Table 2). Conidia were more abundant in the first year than in the other 2 years (Fig. 1e–f). Numbers of conidia ranged from zero (in 75% samples) to 49 conidia cm^{-2} in the traps over inoculum source III in the week between 13 and 20 August 2004 (Fig. 4c).

Maturation of pseudothecia

In the first year, the pseudothecia matured over a long period: the first and last mature pseudothecia were observed on 9 December 2003 and 25 May 2004, respectively; the peak of maturation occurred in January. Maturation lasted for a longer time in the meadow than on the pear leaves (Fig. 2a). In the second year, maturation occurred later over a shorter period of time, especially in the meadow. Most pseudothecia matured between the end of April and mid-June on the pear leaves, whereas in the meadow this period began 1 month later (Fig. 2b). In the third year, pseudothecia on pear leaves and the meadow matured almost in the same period of time between early December and mid-June (Fig. 2c).

Seasonality in ascospore trappings

In plots covered with the pear leaf litter in October 2003 (inoculum type I), ascospores were first trapped in early December (Fig. 3a), according to the presence of mature pseudothecia (Fig. 3a). Ascospores were

Table 2 Results of the ANOVA of repeated measurements applied to the *Pleospora allii* ascospores and *Stemphylium vesicarium* conidia trapped in 3 years, with four types of inoculum sources and 53 7-day samplings per year

Source of variation	Ascospores					Conidia				
	% variance	df source	Df error	*F*	*P*	% variance	df source	df error	*F*	*P*
1. Year	15.9	2	22	110.2	<0.0001	6.4	2	22	25.3	<0.0001
2. Inoculum source[a]	3.8	3	33	45.9	<0.0001	0.2	3	33	1.6	0.202
3. Samplings[b]	15.2	9	100	26.0	<0.0001	32.7	4	42	17.9	<0.0001
4. 1×2[b]	6.6	3	34	40.0	<0.0001	0.3	3	35	1.8	0.157
5. 1×3[b]	28.2	9	101	22.3	<0.0001	49.7	4	40	12.9	<0.0001
6. 2×3[b]	15.5	21	232	14.0	<0.0001	3.6	29	321	1.8	0.008
7. 1×2×3[b]	14.8	28	307	6.9	<0.0001	7.2	28	313	1.5	0.042

[a] See Table 1 for description of the inoculum sources.

[b] For these sources of variation the Mauchly's Test of sphericity was significant, so the degrees of freedom (df) were corrected by the Huynh–Feldt Epsilon.

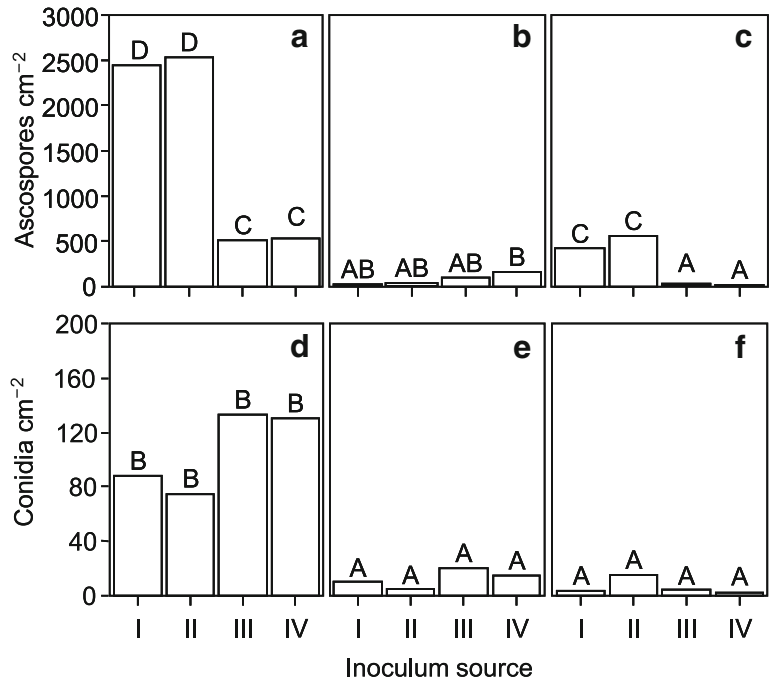

Fig. 1 Total numbers of *Pleospora allii* ascospores and *Stemphylium vesicarium* conidia (of 53 7-day samples, 12 replicates per sample) trapped over 3 years [October 2003 to September 2004 (**a**) and (**d**); October 2004 to September 2005 (**b**) and (**e**); October 2005 to September 2006 (**c**) and (**f**)] from plots with different inoculum sources (see Table 1). *Letters* show significant differences with the LSD Test at $P=0.05$

trapped until the end of August and then became intermittent. Ascospore numbers were high until the end of April, when 95% of the total ascospores trapped in the first year (2,445 ascospores cm^{-2}) had already been trapped; during this period more than 120 ascospores cm^{-2} per week were often obtained, with a maximum of 415 ascospores cm^{-2} per week. Numbers of ascospores were low in May to mid-October when the remaining 5% of the annual ascospores were trapped. In the second year only 18 ascospores cm^{-2} were trapped in 54% samples. Ascospores were first trapped in February 2005, later than in the previous year (Fig. 3a) as the pseudothecia matured later (Fig. 2b). The remaining ascospores were trapped in July–September. In the third year, a total of 427 ascosporesm cm^{-2} were trapped. The most important trapping period was between mid-February and April, with 92% of the total annual ascospores trapped. A few ascospores were also caught between mid-December and mid-January, and between the late October and early November 2006 (Fig. 3a). In the plots of inoculum type II the dynamics of trapped ascospores were very similar to those of inoculum type I (Fig. 3b), with a significant ($P<0.0001$) association between them (a matching coefficient of 0.841 – Table 3).

In the meadow inoculated in October 2003 (inoculum types III and IV), there were two main trapping periods in 2004 (Fig. 3c and d). In the first period (December to March), 23 ascospores cm^{-2} were trapped in total (about 5% of the total number of annual ascospores trapped). In the second period (June–September), there were 480 ascospores cm^{-2} trapped (94%). In the plots that did not receive any further artificial inoculum after October 2003 (inoculum type III) (Fig. 3c), 17 ascospores cm^{-2} were trapped between October 2004 and December 2005, 92 ascospores cm^{-2} in the 13 samples between July and September 2005, and 10 ascospores cm^{-2} in 15 out of the 31 samples between mid-April and mid-October 2006. In the plots where the inoculum was renewed in October 2004 and 2005 (inoculum type IV) (Fig. 3d), ascospores were trapped in 26 out of 35 weeks between February and September 2005 (164 ascospores cm^{-2} in total), and in 21 out of 33 weeks between April and November 2006 (10

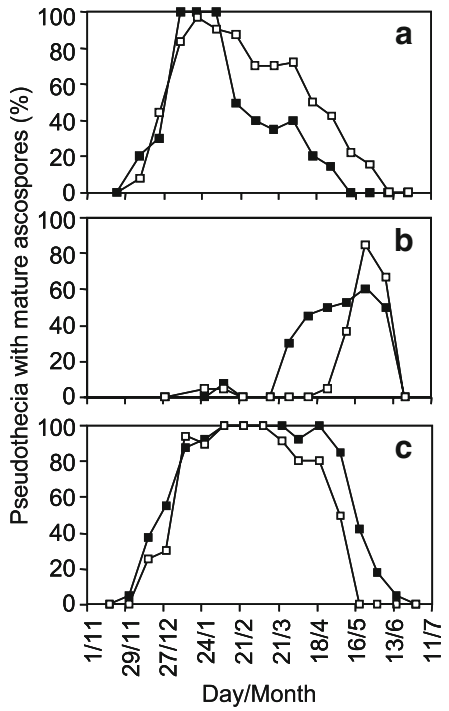

Fig. 2 Frequency of the *Pleospora allii* pseudothecia with mature ascospores formed in pear leaves (*filled square*) and in the meadow (*empty square*) between November and mid-July of 2003/04 (**a**), 2004/05 (**b**), and 2005/06 (**c**). Mature ascospores were observed microscopically in a sample of 20–30 pseudothecia

ascospores cm^{-2}). There was a significant ($P<0.0001$) association (matching coefficient of 0.825) in the periods when ascospores were trapped from inoculum types III and IV (Table 3). The degree of association between the inoculum types with only the meadow inoculated (I and II) and with the pear leaf litter (III and IV) was lower (matching coefficients of 0.706–0.771) though significant (Table 3).

Irrespective of years and inoculum sources, ascospores were trapped in two main periods: December to April and August to October. In the plots with the pear leaf litter (Fig. 3a and b), most ascospores were trapped in the first period, with large differences in ascospore numbers: high in 2003/04, low in 2005/06 and sporadic in 2004/05. The 2 months following the exposure of the inoculated pear leaves were cooler and moister in 2003 than in the other years (Table 4), with an average temperature of 9.9°C, and an average RH of 90.5% (52 days with RH>80%: nearly twice that in 2004 and 2005). Rainfall in 2004 and 2005 was about twice the rainfall in 2003, but the number of rainy days was similar in the 3 years. The period when most ascospores were trapped (December to April) was also moister in 2003/04 (Table 4), with an average RH of 79.6% (66 days with RH>80%) and a total rainfall of 422 mm. In contrast, the number of ascospores trapped was higher in the second period of trapping than in the first one in the plots where the meadow was the only inoculum source (Fig. 3c and d).

Seasonality in conidial trapping

The dynamics of trapped conidia were similar in the 3 years and for the different inoculum sources (Fig. 4). The correlations between trappings in the four inoculum sources were significant ($P<0.0001$): the matching coefficients ranged from 0.812 to 0.888 (Table 3).

Conidia were trapped in the period from April to November, but about 92% of the total number of conidia were caught between July and September (Fig. 4). The numbers of conidia in the first year were higher than in the other 2 years (Fig. 1d–f) even though weather conditions were less favourable for sporulation. In July–September, RH, rainfall and number of rainy days were lower in 2004 than in the other 2 years (Table 3); the number of days with favourable conditions for sporulation estimated according to Rossi et al. (2005b) were 12, 23 and 26 in 2003, 2004 and 2005, respectively.

Pathogenicity of *S. vesicarium* strains

Forty strains of *S. vesicarium* were obtained from the spore traps in 2006: 11 from type I inoculum source, 19 from II, 6 from III and 4 from IV; no strains were obtained from the uninoculated plots (V). All these strains caused brown spot symptoms on the inoculated leaves of cv. Abate Fétel. On the leaves inoculated with the original strain ISA94, the necroses progressed rapidly: disease incidence was 100% 7 days after inoculation (Fig. 5). For most of the 40 *S. vesicarium* strains obtained, the disease progressed similarly as for ISA94 (Fig. 5). For four strains, disease incidence reached 100% only 12–14 days after inoculation; for three strains, the maximum incidence was between 92 and 96%. ANOVA showed that the progress of the disease was not significantly influenced by the inoculum source of the *S. vesicarium* strains and that these strains behaved as the strain ISA94 ($P=0.35$) (Table 5).

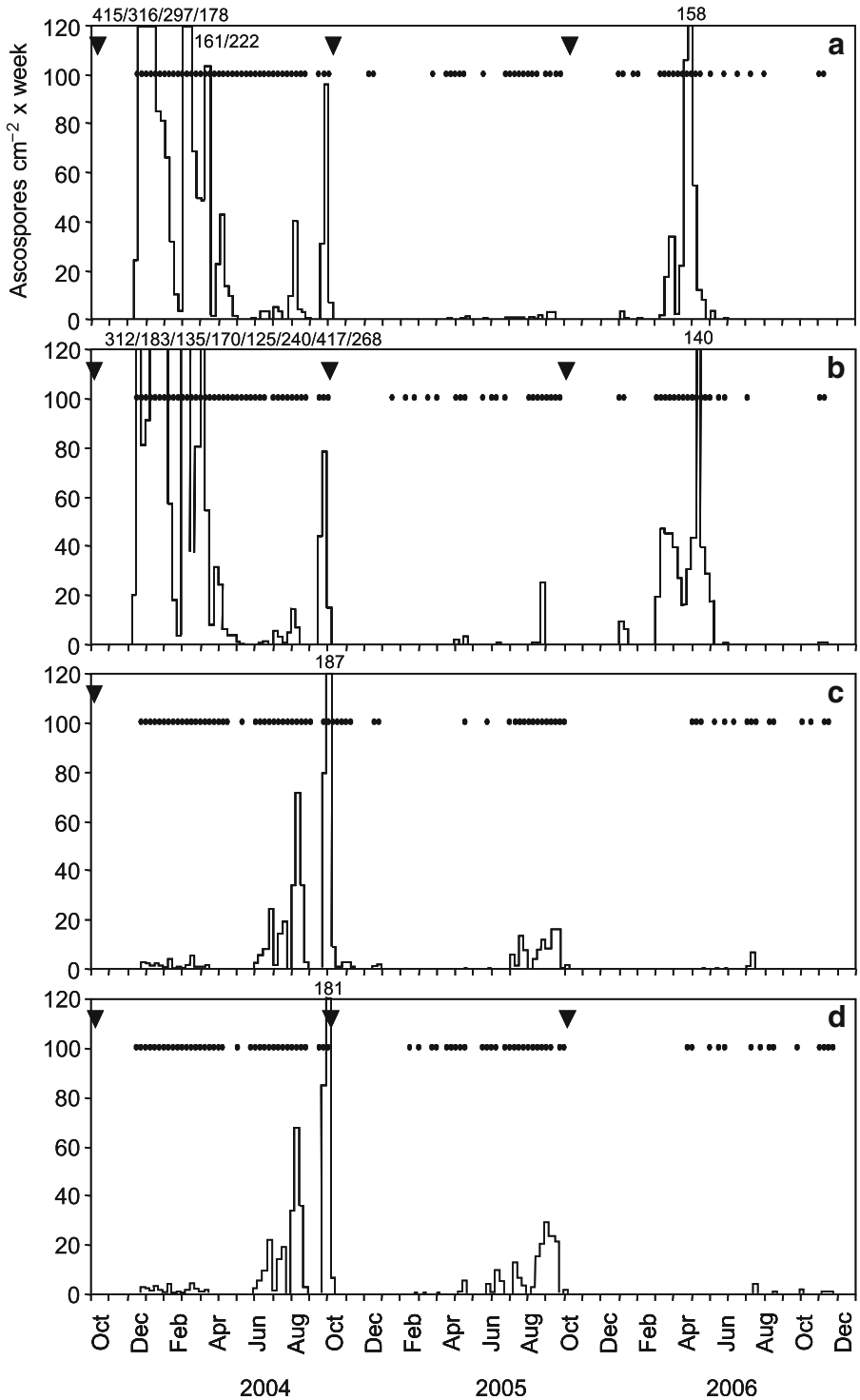

Fig. 3 Numbers of *Pleospora allii* ascospores trapped weekly (means of 12 replicates), in October 2003 to December 2006, from plots with different inoculum sources: **a** corresponds to the inoculum type I (see Table 1), **b** to II, **c** to III, **d** to IV. *Filled triangle* is the time when the inoculum source was inoculated with *Stemphylium vesicarium*; *filled circle* represents a week with positive spore trapping

Discussion

This work concerns sources of the airborne spores of *S. vesicarium* and its teleomorph *P. allii*, which constitute the inoculum for brown spot disease on pear. Previous studies focused on the densities and dynamics of these airborne spores during the season, with no reference to their sources (Picco et al. 1996;

Table 3 Matching between positive and negative trappings of *Pleospora allii* ascospores and *Stemphylium vesicarium* conidia over 170 samples collected between October 2003 and December 2006 in plots with different inoculum sources

Spores	Inoculum source[a]		II		III		IV	
			0[b]	1[b]	0	1	0	1
Ascospores	I	0[b]	75	12	67	20	69	18
		1[b]	15	68	19	64	23	60
			0.841[c]		0.771		0.759	
			76.4[d]		47.6		43.5	
	II	0			66	24	66	24
		1			20	60	26	54
					0.741		0.706	
					37.7		26.8	
	III	0					75	11
		1					17	67
							0.835	
							74.1	
Conidia	I	0	114	17	114	17	117	14
		1	12	27	13	26	11	28
			0.829		0.824		0.853	
			46.7		43.0		57.1	
	II	0			111	15	111	15
		1			16	28	17	27
					0.818		0.812	
					43.5		40.3	
	III	0					118	9
		1					10	33
							0.888	
							80.1	

[a] See Table 1 for description of the inoculum sources.

[b] Spore samples are defined as positive (=1), when at least one spore was trapped, or negative (=0), when no spores were trapped.

[c] Ratio of the simple matching between pairs of data in a sample and total number of samples; positive and negative matches were weighted equally.

[d] χ^2 values for the 2×2 contingency table (all significant with $P<0.0001$).

Table 4 Weather data measured during selected periods of trapping of *Pleospora allii* ascospores and *Stemphylium vesicarium* conidia

Months	Year	Mean temperature °C	Mean RH %	Days with RH>80%	Total rainfall (R) mm	Days with R>0	Days with snow cover
October and November	2003	9.9	90.5	52	101.8	32	0
	2004	12.1	81.5	30	203.8	28	0
	2005	11.4	82.2	24	205.0	35	5
December to April	2003/04	6.2	79.6	66	422.2	60	12
	2004/05	5.8	67.7	25	213.4	53	8
	2005/06	4.8	73.3	35	191.6	68	23
July to September	2004	23.3	57.3	2	121.0	12	0
	2005	22.2	62.7	1	139.8	26	0
	2006	23.1	59.1	4	258.4	29	0

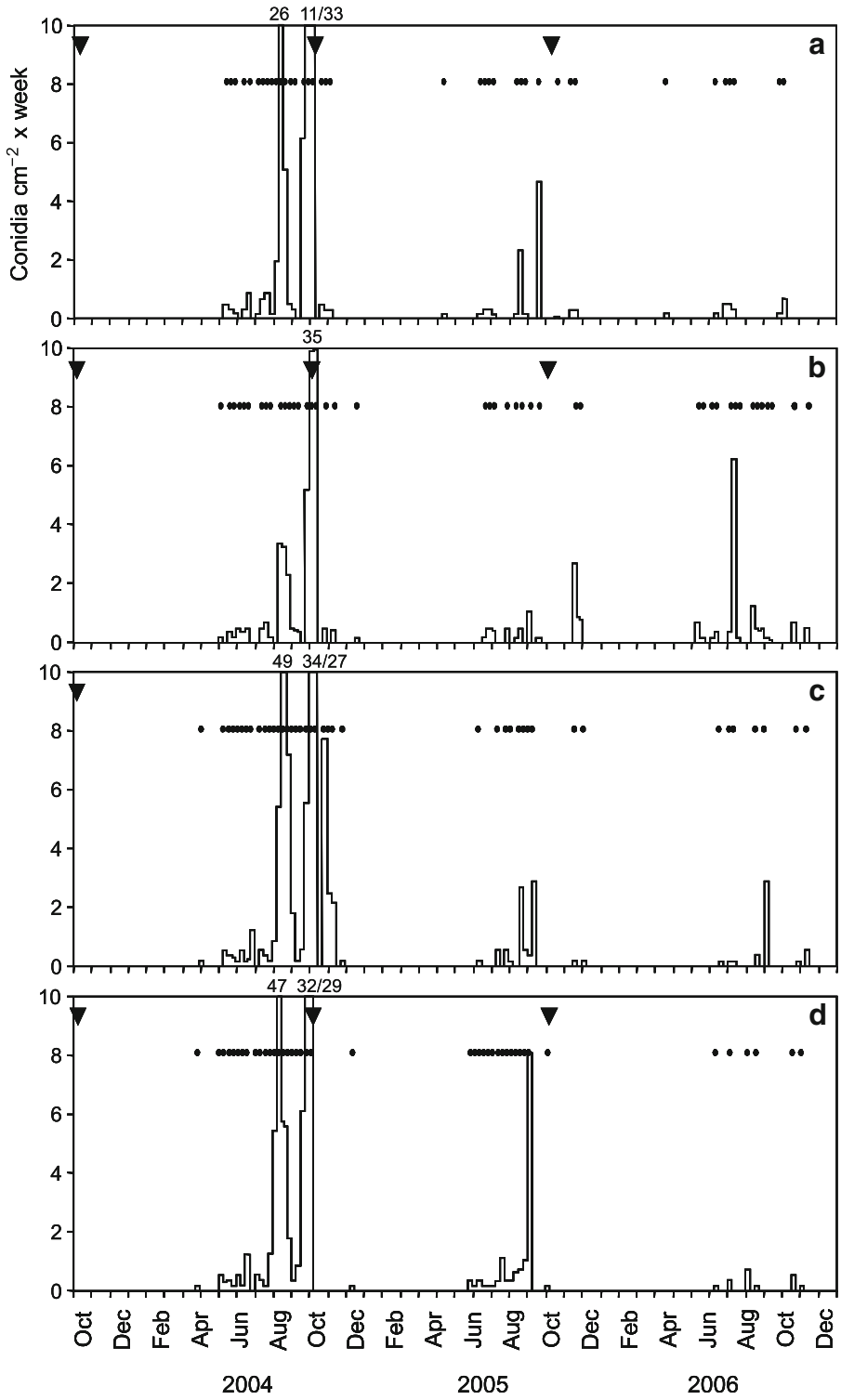

Fig. 4 Numbers of *Stemphylium vesicarium* conidia trapped weekly (means of 12 replicates), in October 2003 to December 2006, from plots with different inoculum sources: **a** corresponds to inoculum type I (see Table 1), **b** to II, **c** to III, **d** to IV. *Filled triangle* is the time when the inoculum source was inoculated with *Stemphylium vesicarium*; *Filled circle* represents a week with positive spore trapping

Maccaferri et al. 2003; Rossi et al. 2005b; Giosuè et al. 2006). Thus the inoculum sources for this disease were not well understood (Llorente and Montesinos 2006). The present results demonstrated that pear leaf litter and the meadow of herbaceous plants covering the ground are potentially important

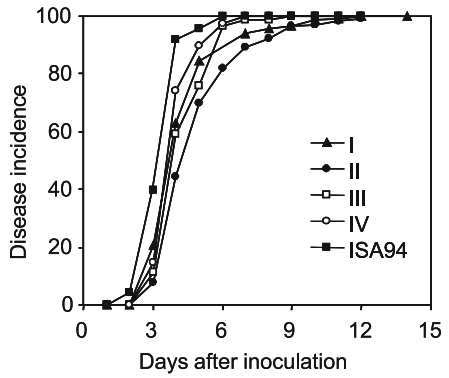

Fig. 5 Comparison between the progress over time of brown spot incidence on pear leaves cv. Abate Fétel inoculated with *Stemphylium vesicarium*, strain ISA94 and strain from different inoculum sources (see Table 1)

sources of inoculum, as previously speculated by Rossi et al. (2005a).

On average, trappings of ascospores were about five times higher from the plots with the pear leaf litter than from those with the meadow only. Nevertheless, in the latter case, an annual average of 218 ascospores were trapped cm^{-2} of the trapping surface. On the contrary, the conidial numbers caught were similar for the different types of inoculum sources, with an annual average of 42 conidia cm^{-2}. These spore densities should be considered with caution, because the leaf litter and the meadow had been artificially inoculated with the fungus, and therefore the level of the resulting inoculum would be higher than in an orchard subjected to natural infections of brown spot.

The dynamics of both conidia and ascospores were consistent between different types of the inoculum source and years. The conidial season lasted from April to November, with most conidia caught between July and September. The presence of airborne conidia of *S. vesicarium* during the warmer period of the year was in substantial agreement with previous studies (Hausbeck et al. 1999; Suheri and Price 2001; Prados-Ligero et al. 2003; Rossi et al. 2005b).

The ascospore season was much longer, with two main periods: December to April, and August to October. The former was the most important in the presence of the pear leaf litter, while the latter prevailed for the meadow. The presence of the sexual stage of *P. allii* in the cooler months has been previously documented in asparagus and onion or leek debris affected by purple spot and leaf block, respectively (Aveling 1993; Hausbeck et al. 1999; Suheri and Price 2001; Prados-Ligero et al. 1998, 2003), and also in fallen pear leaves affected by brown spot (Llorente and Montesinos 2004; Llorente et al. 2006).

The second period of ascospore trapping has not been reported previously for this pathogen on pears or other hosts. A possible reason is that in all these cases, the production of ascospores was investigated for a restricted period of time related to their possible role in infecting host plants (Hausbeck et al. 1999; Prados-Ligero et al. 1998, 2003; Llorente and Montesinos 2004; Rossi et al. 2005b). Present data were obtained throughout the year and were in agreement with the presence of ascospores belonging to *Pleospora* spp. in non-crop environments (Vittal and Krishnamoorthi 1988; Mitakakis and Guest 2001; Gioulekas et al. 2004; Díez-Herrero et al. 2006). For instance, *Pleospora* spores were present on 71% of the days of the year in the area of Madrid, with peaks in March, April, September and October (Díez-Herrero et al. 2006). This similarity is stressed by

Table 5 Comparison between the progression rates of brown spot incidence on pear leaves of cv. Abate Fétel inoculated with *Stemphylium vesicarium* strains from different inoculum sources and the strain ISA94

Type of inoculum[a]	No. of strains	Rate of disease progression[b]			
		Mean	Standard error	Min.	Max.
I	11	1.27	0.242	0.47	2.29
II	19	1.11	0.225	0.34	3.17
III	6	1.20	0.111	0.66	1.39
IV	4	1.67	0.367	0.91	2.40
ISA94	4	1.92	0.162	1.44	2.22

[a] See Table 1 for description of the inoculum sources.

[b] Rates were calculated as the slope of the regression lines fitting the gompit transformation of disease incidence to time, where gompit is the linearizing transformation of the Gompertz function: $\text{gompit}=-\ln[-\ln(y)]$.

the fact that the ascospores we trapped in the second period were from the meadow plants. Therefore, the saprophytic colonisation by *S. vesicarium* of dead plant material from the meadow could act as an important source of inoculum.

Results from the present work contribute to a better understanding of the role of ascospores in the life cycle of the pathogen. It has been assumed that these spores initiate the saprophytic colonisation of the dead material in the meadow during the winter and spring (Rossi et al. 2005a; Llorente and Montesinos 2006) with new sexually-derived genotypes, which maintains their infectivity on pear (Llorente and Montesinos 2006; Rossi et al. 2006). This study has demonstrated that this process begins in autumn and contributes to a significant proportion of colonisation of the meadow and the consequent production of abundant conidia. In fact, the weather conditions from October 2003 to April 2004 favoured early maturation of pseudothecia that continued for long time, with early, long and high production of ascospores between December and April, so that in summer 2004, the numbers of conidia were high. In autumn 2004 to spring 2005, the weather conditions were less favourable, pseudothecia matured later and for a shorter period with low numbers of ascospores produced, so that the production of conidia in summer 2005 was lower than in 2004, despite the fact that weather conditions were more favourable for asexual sporulation in 2005. Therefore, the inoculum production in summer depends on the colonisation rate of the dead material on the orchard floor, which in turn depends on the weather conditions from autumn to spring, as well as the current summer conditions (Rossi et al. 2005b; Giosuè et al. 2006).

This study also demonstrated, for the first time, that the pathogen is capable of colonizing the orchard floor persistently. The plots with a wildflower meadow inoculated in early October 2003 with the virulent stain ISA94 of *S. vesicarium* produced airborne spores until the end of the experiment: the last conidia were trapped on 20–27 October 2006, and the last ascospores on 3–10 November 2006. Considering that: (1) there were no other sources of *S. vesicarium* inoculum from pears in the vicinity; (2) the spore traps were placed a few centimetres above the ground, thus the ingression of spores from the other plots, which were separated by 1 m wide bands, may be considered not important; (3) neither ascospores nor conidia were trapped from the uninoculated meadow; (4) the fungal strains obtained from the airborne propagules in 2006 were all able to cause brown spot on pear leaves as did strain ISA94, it can be reasonably concluded that the airborne spores trapped were produced from the meadow that had been inoculated in 2003.

In conclusion, the present work demonstrates that the orchard ground is a permanent source of both sexual (ascosporic) and asexual (conidial) inoculum for brown spot of pear. The implications of this information for disease control concern the possibility of reducing the amount of inoculum by managing the orchard ground. Recently, sanitation measures for the pear orchard ground were investigated with the aim of reducing overwintering sexual inoculum (Llorente et al. 2006). Further work should be aimed at verifying the effectiveness of these measures in reducing the inoculum all year long, particularly the asexual inoculum during epidemics.

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